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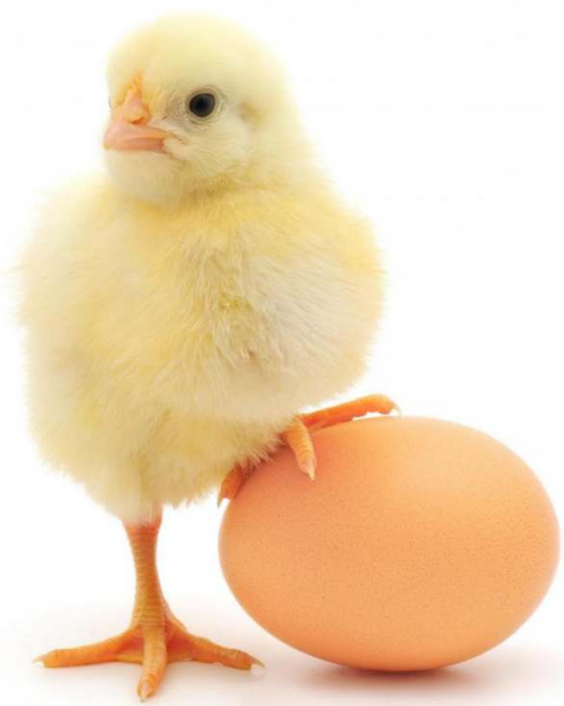

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Research Article
Effect of Dietary Calcium Intake and Limestone Solubility on Egg Shell Quality and Bone Parameters for Aged Laying Hens

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Abstract

Background and Objective: Calcium requirement for laying hens need updated information as the genetic changes every year. Calcium are usually increased when laying hens mature in age; therefore, the objective of this study was update the calcium requirement in aged hens for optimum eggshell quality and bone strength. **Methodology:** Two experiments of 10 week experimental periods using 240 and 320 hens for experiment 1 and 2, respectively were conducted to study the effects of calcium intake and solubility on egg shell quality and bone status in laying hens 77-94 weeks age. Leghorn hens were randomly assigned into a 2×4×5 factorial arrangement of treatments (2 Ca sources, 4 different limestone sizes and 5 predicted calcium intake levels). **Results:** The findings showed that Shell Weight per Unit of Surface Area (SWUSA), egg Specific Gravity (SG), bone ash concentration and bone breaking force were significantly improved by the reduction of Limestone Solubility (LS) and higher Daily Calcium Intake (DCI) ($p<0.01$). The highest SWUSA was obtained from hens fed 4.89 g DCI with 30.1-39.8% LS. The SG showed a similar trend to SWUSA. The greatest bone-breaking force was for hens fed 5.89 g DCI and 33.5% LS while the highest bone ash concentration was obtained from hens fed 5.89 g DCI and 30.1% LS. **Conclusion:** The results suggested that lower LS (30.1-39.8% for shell and 30.1-33.5% for bone breaking force) with a higher daily calcium intake (3.94-4.89 g and 5.89 g hen^{-1} day^{-1} for maximum shell quality and bone strength, respectively) should be recommended for aged laying hens.

Key words: Hen, calcium, requirement, limestone, solubility

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Few studies have been performed during the last years on particle size and calcium solubility for laying hens. The National Research Council[1] have consistently increased the calcium (Ca) requirement of laying hens on each new publication for more than 40 years[2,3]. The trends of NRC consistently increasing the Ca requirement for layers either as percentage of feed or as gram per day was stopped in their previous study of NRC[1] when the council suggested the Ca intake of layers could be reduced from 3.75 (NRC)[4] to 3.25 g hen^{-1} day^{-1}. Although the suggested daily requirement for layer Ca was reduced by NRC[1], several study groups have continued to show that Ca intake in current layers above 3.75 g hen^{-1} day^{-1} improves shell quality[2,5-10]. Many factors can influence the dose response of Ca for layers. The age of hen and different Ca sources may have a large impact on Ca requirement for shell quality and bone status. Extrapolation of the data of Cheng and Coon[11] with layers consuming different particle sizes and daily intakes of Ca shows layers produced quality egg shells (based on specific gravity values of 1.080) at different intakes of Ca. Two grams intake of calcium carbonate provided in large particles (U.S. screen size of 6) of limestone produced an average specific gravity value of 1.080 whereas hens consuming very fine limestone (U.S. screen size of 100) required 3 g of daily Ca intake to reach the same standard. Since the intake study was established for a short 6 week period, the researchers are not suggesting that layers fed large particle limestone only need 2 g of Ca per day for maximum performance and bone status but the data showed that Ca: egg shell response is different depending upon the particle size. A decrease in eggshell quality with age has been well documented[12] and may be caused by decreased ability to absorb Ca from the digestive system and less mobilization of Ca from the medullary bones due to aging[13]. The beneficial effects of feeding larger particle coarse Ca has been demonstrated by a number of researchers[1,14-16]. The larger particle Ca source may increase egg shell quality through a prolonged retention of Ca source in the gizzard, hence increased Ca solubilization and bioavailability[3,14,17] connected with the timing of optimum utilization of Ca for egg shell formation during the dark period. Besides age and calcium particle size, the large variation for suggested Ca requirement for layers is caused by many factors: stage of lay, cumulative shell mass produced, environmental temperature, disease status such as infectious bronchitis, molting and solubility of Ca from calcium carbonate source. The current study compared two calcium carbonate sources, four particle sizes and five levels of Ca intake to investigate the effect on performance, shell quality and bone status for older layers in first cycle (Experiment 1, 77 weeks of age) and for molted layers in second cycle (94 weeks of age).

MATERIALS AND METHODS

Two experiments were conducted for a 10-week period in three environmentally controlled rooms. In Experiment 1 (240 hens; 77 weeks of age) and Experiment 2 (320 hens; 94 weeks of age) H&N Leghorn hens were randomly assigned into a 2×4×5 factorial arrangement of treatments with 2 different Ca sources, 4 different limestone sizes (average United States Standard Screen Numbers: 7, 12, 25 and 60) and 5 predicted calcium intake levels (2, 3, 4, 5 and 6 g day^{-1} hen^{-1} with 115 g feed intake/day/hen). Each treatment had six replicates in experiment 1 and eight in experiment 2. Hens in laying stage were selected from a large flock population and individually caged and provided with feed (Table 1) and water for *ad libitum* access. The hens used in the two experiments were from the same hatch. The layers used in experiment 1 were not molted while the hens in experiment 2 were molted at 64 weeks of age. The solubility value of each particle size of limestone (Table 2) was determined by the Weight Loss

Table 1: Experimental diet for laying hens

Ingredients	Percentage
Yellow corn	51.26
Soybean meal CP47	26.61
Dicalcium phosphate	2.10
Salt	0.22
Sodium bicarbonate	0.28
DL-methionine	0.14
*Vitamin premix	0.08
**Mineral premix	0.06
Animal fat	4.44
***Limestone source	Adjusted to 100
***Silica	Adjusted to 100

*Vitamin premix supplied the following per kilogram of diet: vitamin A, 4400 IU; vitamin D3, 2200 IU; vitamin E, 11 IU; vitamin K, 0.53 mg; niacin, 23 mg; riboflavin, 3.1 mg; folacin, 0.2 mg; pantothenic acid, 4.1 mg; vitamin B_{12}, 004 mg. **Mineral premix supplied the following in milligrams per kilogram of diet: Fe, 15; Zn, 34; Mn, 54; Cu, 2; I, 0.6. ***The percentages of limestone and silica were adjusted to provide 2, 3, 4, 5, 6 g/115 g feed, respectively

Table 2: Solubility of limestone sources using MWLM and WLM methods#

	Solubility (%)	
*Size	Source 1 (%)	Source 2 (%)
7	30.1 (8.5)	33.5 (9.3)
12	39.8 (11.3)	45.7 (13.4)
25	49.6 (14.9)	52.3 (15.2)
60	58.7 (17.5)	63.2 (18.0)

#Numbers in parenthesis were determined using WLM method. *Average screen number: screen number where 50% of limestone passed through and 50% was retained by the screen. Screen numbers 7, 12, 25 and 60 correspond to 2.75, 1.82, 0.74 and 0.26 mm, respectively

Method (WLM)[18] and by the modified WLM (MWLM)[19] that is different in acidity and volume of the solution (200 mL of 2 N HCl in MWLM vs. 100 mL of 0.1 N HCl in WLM). Egg production was recorded daily and eggs laid on three consecutive days by each hen were collected and weighed every week. Egg shell weight, Shell Weight per Unit of Surface Area (SWUSA), Specific Gravity (SG) and feed intake were measured biweekly. Surface area of an egg was calculated by the method of Paganelli *et al.*[20] and SG was measured using 9 saline solutions ranging from 1.060-1.100 in the specific gravity. The calcium content of the basal diet and the different limestone was analyzed by an atomic absorption spectrophotometer before mixing.

The hens were sacrificed by cervical dislocation at the end of the 10 week feeding period. Right tibia bones were removed and stored at -20°C until tested for the various bone parameters. Bone volume was taken by a weight change in water using the method by Zhang and Coon[21] in which the weight change of a bone (weight in the air-weight in the water) was assumed to be the volume assuming the specific gravity of water is 1.0 g cm^{-3}. Bone ash weight was obtained after incineration at 600°C for 24 h. Bone ash concentration was calculated by Eq. 1:

$$\text{Bone ash concentration} = \frac{\text{Bone ash weight}}{\text{Volume}} \tag{1}$$

Bone breaking force was measured by an Instron Testing Machine (Model 1122; Canton, MA 02021). Tibia bones were supported by a fulcrum with 7 cm width. A probe with 1.4 cm length and 0.3 cm at the base was attached to a 50 kg load cell with a crosshead speed of 200 mm/min.

Statistical analysis: Data was analyzed by using SAS, statistical analysis software[22]. A completely randomized design was used. When the effects were significant, means were separated using Duncan's range test at $p \leq 0.05$. Data was analyzed by ANOVA is presented as mean with overall SEM and superscripts[a-b] letters to show statistical differences for $p \leq 0.05$. The average measurements for each variable were used in analyzing data. Hens that were going through natural molting during the experiments were excluded from the analysis. Limestone solubility instead of source and particle size was used in data analysis. It means that limestone source (2)× particle size (4), a total of 8 treatments will be presented for each of the parameters evaluated. It has been reported that regressed shell quality and bone parameter traits for layers relates better to limestone Ca solubility than limestone particle size[18]. All procedures regarding the use of live animals in this study were carried out in accordance with the Animal Use Protocol 03008, which was approved by the University of Arkansas Institutional Animal Care and Use Committee.

RESULTS AND DISCUSSION

Solubility of the 2 calcium sources determined using MWLM and WLM methods were summarized in Table 2. Hereafter, solubility results and discussions are based using MWLM data unless specified. The hens used in experiment 1 were significantly different from those in experiment 2 in body weight, egg weight, egg mass, egg production, egg shell weight, SWUSA, daily feed intake but not in egg specific gravity, feed conversion (feed consumption/egg mass), bone breaking force and bone concentration (Table 3). Hens in experiment 2 had larger body size, better shell quality (SWUSA), higher egg production, egg weight and feed consumption and produced more egg mass and egg shell output ($p<0.05$) compared with those in experiment 1 (Table 4). Although the hens in experiment 1 were different from those in experiment 2 in body size and performance, no interaction effects were found between experiments×Ca dietary level, experiment×limestone solubility and experiment×Ca dietary level×limestone solubility on performance, egg shell quality and bone parameters (Table 3, 4). Thus, the data from the two experiments were pooled together in the evaluation of these variables.

Actual daily Ca intake was calculated based on feed consumption and calcium dietary level. The five calcium dietary levels (2, 3, 4, 5 and 6 g/115 g feed) were equivalent to 1.98, 2.96, 3.94, 4.89 and 5.89 g of actual daily calcium intake. Dietary Ca level and limestone solubility did not affect egg weight, egg mass, egg production, feed consumption and feed conversion (Table 3, 5). Those results are consistent with previous reports[2,15,18,23-24]. It has been suggested by Miller[25] that layers feeding high levels of Ca may reduce feed intake and possibly regulate egg weight although the majority of data does not support this belief. The SWUSA and egg specific gravity were significantly increased with higher Ca levels and lower limestone solubility. The highest SWUSA and specific gravity were obtained at 5 g hen^{-1} day^{-1} predicted Ca intake (4.89 g/hen/day actual Ca intake) and 30.1% limestone solubility. However, the differences in SWUSA and specific gravity were not significant for the layer groups fed 4, 5 or 6 g day^{-1}. The SWUSA was increased with a decrease in

Table 3: Probabilities of treatments on various parameters

Source of variation	Body weight	Egg weight	Egg mass	Egg production	Egg shell weight	Specific gravity	SWUSA	Daily feed intake	Feed conversion	Tibia bone breaking force	Tibia bone ash concentration
						Probabilities					
EXP	0.0004	0.0158	0.0003	0.0149	0.0029	0.1702	0.0125	0.0001	0.8083	0.4862	0.4216
SLB	0.7488	0.9561	0.7440	0.5149	0.0492	0.0473	0.0073	0.1985	0.3887	0.0380	0.3293
LVL	0.3224	0.6086	0.3793	0.6841	0.0001	0.0001	0.0001	0.6723	0.0903	0.0001	0.0001
SLB×EXP	0.2682	0.9657	0.7261	0.7347	0.4190	0.3658	0.1402	0.9427	0.9349	0.8971	0.7417
LVL×EXP	0.4460	0.1358	0.1965	0.9518	0.3788	0.4339	0.6791	0.4490	0.3152	0.5124	0.4325
SLB×LVL	0.6881	0.3822	0.2269	0.1201	0.2005	0.3424	0.1795	0.3511	0.4792	0.1137	0.8345
SLB×LVL	0.9351	0.6074	0.8624	0.7034	0.4844	0.1190	0.2212	0.3882	0.5148	0.6219	0.4418

EXP = Experiment, SLB = Solubility and LVL = Ca dietary level

Table 4: Hen performance, various shell quality traits and tibia parameters of hens in the two experiments

Experiment	Body weight (g)	Egg weight (g)	Egg mass (g day^{-1})	Egg production (%)	Egg shell weight (g)	Specific gravity	SWUSA (mg cm^{-2})	Feed consumption (g hen^{-1} day^{-1})	Feed conversion (g g^{-1})	Tibia bone breaking force (kg)	Tibia bone ash concentration (g cm^{-3})
1	1786	62.7	50.7	80.8	5.26	1.0765	70.25	109.9	2.18	9.184	0.4449
2	1855	64.2	53.1	82.8	5.42	1.0763	71.25	113.9	2.16	9.260	0.4460
SEM	13.83	0.331	0.391	0.570	0.043	0.0005	0.464	0.586	0.016	0.184	0.008

limestone Ca solubility but no significant difference in SWUSA was found when solubility was equal to or less than 39.8%. Specific gravity was also increased along with the decrease in solubility. The trend of increasing specific gravity with decreasing limestone solubility was similar with that of SWUSA (Table 6). The data showed that a minimum Ca intake of 3.94 g hen^{-1} day^{-1} or above up to 4.89 g Ca/hen/day with a limestone solubility of 30.1-39.8% as determined by MWLM was appropriate for maximum shell quality for older layers. Pelicia *et al.*[26] found that 5.5 g hen^{-1} day^{-1} (dietary calcium: 4.5%) produced better eggshell quality for older hens, Hisex Brown 90-108 weeks of age. Utilized hens that were older than the hens used in the present experiment but provide a trend that older hens may need higher levels of Ca in their diet compared to young hens[26]. These values indicate that daily Ca intake requirement for older laying hens may be higher along with a lower percentage limestone Ca solubility compared to young hens for shell quality. Cheng and Coon[11] recommended 11-14% limestone solubility determined by WLM. Cheng and Coon[18] determined 3.75 g hen^{-1} day^{-1} in calcium intake for 36 week old hens. A limestone solubility of 11-14% determined by the WLM is comparable to a limestone solubility of 39-48% by MWLM. The results from experiment 1 and experiment 2 with older layers confirm reports that Ca intake above 3.75 g hen^{-1} day^{-1} may improve shell quality. The best eggshell quality with 4.51 g hen^{-1} day^{-1} of Ca intake at 56-57 week old laying hens from which calcium requirement fits in between the values required for young and old hens[27].

Bone breaking force was significantly affected by dietary Ca level ($p<0.05$). Both, bone ash concentration and bone breaking strength were increased with the increase in daily Ca intake. The highest bone parameter values were observed with hens fed 6 g Ca/day (actual intake of 5.89 g hen^{-1} day^{-1}). No response plateau with Ca intake was found for either of the bone parameters used in present study (Table 7). The results showed a higher daily Ca requirement for maintaining bone status than for egg shell quality which is in agreement with previous study with younger laying hens (36 week of age)[11]. Lower limestone solubility significantly increased bone breaking force ($p<0.05$) of the older layers and a similar trend was found for bone breaking strength and bone ash concentration. The range of 30.1-33.5% (as determined by MWLM) in solubility seems to provide satisfactory bone ash concentration (Table 7). This range is again lower than the solubility range of 11-14% by WLM (equivalent to a range of 39-48% by MWLM) recommended for bone ash concentration in young hens (36 weeks of age)[1]. Beneficial effects of limestone with low *in vitro* solubility on egg shell quality and

Table 5: Effect of source, solubility and calcium level of diet on egg production, egg weight, egg mass, feed consumption and feed conversion

Variables	Egg production (%)	Egg mass (g day^{-1} hen^{-1})	Egg weight (g)	Feed conversion (feed/egg mass)	Feed consumption (g day^{-1} hen^{-1})
Solubility (%)					
63.2	80.600	51.500	63.900	2.22	114.200
58.7	81.200	51.300	63.100	2.20	112.100
52.3	83.900	53.100	63.200	2.12	111.800
49.6	81.900	52.500	64.100	2.19	114.500
45.7	83.500	53.300	63.800	2.16	113.900
39.8	82.000	52.000	63.500	2.15	111.200
33.5	81.800	52.000	63.600	2.13	110.500
30.1	80.800	51.800	64.200	2.18	112.100
SEM	01.057	0.760	0.083	0.031	01.132
Dietary Ca level (Ca g/115 g feed)					
2	81.300	51.200	62.800	2.22	113.100
3	81.000	51.700	63.900	2.21	113.600
4	82.700	52.800	63.900	2.14	112.400
5	82.900	52.600	63.500	2.16	112.400
6	82.100	52.300	63.700	2.13	112.700
SEM	0.871	0.610	0.067	0.025	0.909

Table 6: Effect of limestone solubility and Ca intake on shell quality traits for 77-94 week old hens

Variables	SWUSA[#] (mg cm^{-2})	Specific gravity
Solubility (%)		
63.2	68.29^{c}	1.0749^{b}
58.7	69.32^{c}	1.0753^{b}
52.3	69.90^{bc}	1.0753^{b}
49.6	70.65^{bc}	1.0760^{ab}
45.7	70.16^{bc}	1.0759^{ab}
39.8	72.52^{ab}	1.0761^{ab}
33.5	72.33^{ab}	1.0769^{ab}
30.1	74.10^{a}	1.0782^{a}
SEM	0.888	0.0009
Dietary Ca level (Ca g/115 g feed)		
2	64.67^{c}	1.0704^{c}
3	69.33^{b}	1.0765^{b}
4	72.00^{a}	1.0775^{ab}
5	73.70^{a}	1.0783^{a}
6	72.21^{a}	1.0777^{ab}
SEM	0.7133	0.0007

[#]SWUSA = shell weight per unit surface area. $^{a-c}$Means within each column and variable with no common letters are significantly different (p<0.05)

Table 7: Effect of treatments on tibia parameters for 77-94 week old hens

Variables	Bone ash concentration (g mL^{-1})	Bone-breaking force (kg)
Solubility (%)		
63.2	0.4282	8.68^{bc}
58.7	0.4423	8.51^{c}
52.3	0.4357	9.24^{abc}
49.6	0.4394	9.28^{abc}
45.7	0.4306	9.54^{ab}
39.8	0.4605	9.31^{abc}
33.5	0.4600	9.88^{a}
30.1	0.4676	9.67^{ab}
SEM	0.0140	0.314
Dietary Ca level (Ca g/115 g feed)		
2	0.3988^{d}	7.45^{c}
3	0.4307^{c}	9.04^{b}
4	0.4496^{bc}	9.72^{ab}
5	0.4643^{ab}	9.86^{a}
6	0.4862^{a}	10.15^{a}
SEM	0.0108	0.248

$^{a-d}$Means within each column and variable with no common letters were significantly different (p<0.05)

bone status may be contributed by its longer retention in gizzard, higher solubility *in vivo*[28-29] slower Ca releasing rate which may promote more efficient Ca absorption.

Wide differences in the recommended Ca intake for laying hens exists and ranges from 2.7 up to 6.2 g day^{-1} hen^{-1} in study. Several factors may have been involved in causing such a dilemma, such as the differences existed among studies in stage of laying, age, strain and environmental temperature. The Ca solubility may have also played a role because of its strong effect on Ca *in vivo* solubilization, thus affecting the calcium retention[28-29].

The calcium required by laying hen may be affected by egg production, age, stage of production, limestone source and particle size or solubility. Environmental temperature may also have an impact on the optimum solubility for shell quality and bone parameters[19]. The suggested Ca intake of 3.25 g hen^{-1} day^{-1} by NRC[1] for an entire laying cycle may not be robust enough to take into account these factors and may limit the application to specific conditions. Models are needed to relate Ca to shell and bone measurements and develop a better understanding of how biological and environmental factors change these responses so that more accurate recommendations can be made under different conditions.

In the present study, to maximize shell quality in older layers the data shows the layer needs 3.94-4.89 g Ca intake/hen/day when providing a calcium carbonate source with a solubility range of 30.1-39.8% (by MWLM). A higher Ca daily intake (6 g Ca intake from calcium carbonate source with solubility of 30.1-33.5%) with lower solubility is suggested for maintaining maximum bone strength in the older layer compared to younger layers. It should be noted that the actual optimal calcium intake for older layers may be lower than the level shown in present study. Hens that stopped laying were excluded from the shell quality and bone status evaluation process in both experiment 1 and experiment 2. Hens that are not laying reduce their feed consumption and thus decrease the average calcium intake for the flock. The proportion of non-laying hens in a flock would be dependent upon factors such as age, stage of lay, environmental temperature, management, nutrition etc., hence; no estimate of optimal calcium intake in a dose response study can be expected without excluding non-layers. The knowledge of feed intake for hens that are laying is thus essential in feed formulation. An average feed intake is unlikely to provide adequate information for an aged flock due to the increasing number of non-laying hens compared to that of young flocks. Recently, Roland and group[24] suggested 32 week hens need 4.2 g hen^{-1} day^{-1}. According to the researchers, calcium intake of 3.25 g day^{-1} hen^{-1} (NRC)[1] is not sufficient to support the performance variables such as egg shell quality, bone status and maybe egg production in both young and old laying hens.

CONCLUSION

It can be concluded that aged non-molted laying hens (77 weeks) or older molted second cycle layers (94 weeks) require lower Ca solubility and higher Ca intake compared to relative younger laying hens (36 weeks old) to maximize shell quality and bone status. The amounts 3.94-4.89 g Ca intake/hen/day from calcium carbonate source with a solubility range of 30.1-39.8% (by MWLM) is recommended for older layers. The NRC recommendation of 3.25 g Ca/hen/day may not be sufficient to support the performance variables such as egg shell quality and bone status in older non-molted laying hens or older molted second cycle laying hens.

SIGNIFICANCE STATEMENTS

- Egg shell quality is a very important trait in the laying egg industry because it signifies the production of a useable egg. When the egg shell quality is poor, microbial contamination could occur resulting in a hazard for human health; therefore, the evaluation of nutrients to improve egg shell quality is vital
- Calcium is the nutrient associated with eggs shell quality, consequently, Ca source, solubility, particle size and other features need constant evaluation in laying hens
- This study provides suggested amounts of Ca intake: 3.94-4.89 g Ca intake/hen/day from calcium carbonate source with a solubility range of 30.1-39.8% (by MWLM) for older laying hens. These very specific recommendations are very valuable for the laying egg industry which can be put into practice

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REFERENCES

1. NRC., 1994. Nutrient Requirements of Poultry. 9th Edn., National Academy Press, Washington, DC., USA., ISBN-13: 9780309048927, Pages: 155.
2. Keshavarz, K. and S. Nakajima, 1993. Re-evaluation of calcium and phosphorus requirements of laying hens for optimum performance and eggshell quality. Poult. Sci., 72: 144-153.
3. Zhang, B., 1994. Studies of Ca and P nutrition in laying hens. M.Sc. Thesis, University of Minnesota, Minnesota.
4. NRC., 1984. Nutrient Requirements of Poultry. 8th Rev. Edn., National Academy Press, Washington, DC., USA.
5. Atteh, J.O. and S. Leeson, 1985. Response of laying hens to dietary saturated and unsaturated fatty acids in the presence of varying dietary calcium levels. Poult. Sci., 64: 520-528.
6. Keshavarz, K., 1987. Interaction between calcium and phosphorus in laying hens. Nutr. Rep. Int., 36: 9-20.
7. Gleaves, E.W., F.B. Mather and M.M. Ahmad, 1977. Effects of dietary calcium, protein and energy on feed intake, egg shell quality and hen performance. Poult. Sci., 56: 402-406.
8. Roland, Sr. D.A., B.L. Damron and R.H. Harms, 1977. Specific gravity of eggs as influenced by dietary calcium and time of oviposition. Poult. Sci., 56: 717-719.Roland, D.A., 1986.
9. Egg shell quality III: Calcium and phosphorus requirements of commercial Leghorns. World's Poult. Sci. J., 42: 154-165.
10. Clunies, M., D. Parks and S. Leeson, 1992. Calcium and phosphorus metabolism and eggshell formation of hens fed different amounts of calcium. Poul. Sci., 71: 482-489.
11. Cheng, T.K. and C.N. Coon, 1990. Effect of calcium source, particle size, limestone solubility *in vitro* and calcium intake level on layer bone status and performance. Poult. Sci., 69: 2214-2219.
12. Abe, E., H. Horikawa, T. Masumura, M. Sugahara, M. Kubota and T. Suda, 1982. Disorders of cholecalciferol metabolism in old egg-laying hens. J. Nutr., 112: 436-446.
13. Petersen, C.F., 1965. Factors influencing egg shell quality-a review. World's Poult. Sci. J., 21: 110-138.

14. Scott, M.L., S.J. Hull and P.A. Mullenhoff, 1971. The calcium requirements of laying hens and effects of dietary oyster shell upon egg shell quality. Poult. Sci., 50: 1055-1063.
15. Brister, Jr. R.D., S.S. Linton and C.R. Creger, 1981. Effects of dietary calcium sources and particle size on laying hen performance. Poult. Sci., 60: 2648-2654.
16. Keshavarz, K. and C.C. McCormick, 1991. Effect of sodium aluminosilicate, oyster shell and their combinations on acid-base balance and eggshell quality. Poult. Sci., 70: 313-325.
17. Rao, K.S., D.A. Roland Sr., J.L. Adams and W.M. Durboraw, 1992. Improved limestone retention in the gizzard of commercial Leghorn hens. J. Applied Poult. Res., 1: 6-10.
18. Cheng, T.K. and C.N. Coon, 1990. Comparison of various *in vitro* methods for the determination of limestone solubility. Poult. Sci., 69: 2204-2208.
19. Zhang, B.F. and C.N. Coon, 1997. Improved *in vitro* methods for determining limestone and oyster shell solubility. J. Applied Poult. Res., 6: 94-99.
20. Paganelli, C.V., A. Olszowka and A. Ar, 1974. The avian egg: Surface area, volume and density. Condor, 76: 319-325.
21. Zhang, B. and C.N. Coon, 1997. The relationship of various tibia bone measurements in hens. Poult. Sci., 76: 1698-1701.
22. SAS., 2012. SAS User's Guide Statistics, Version 9.3. SAS Institute Inc., Cary, NC., USA.
23. Hamilton, R.M.G., R.W. Fairfull and R.S. Gowe, 1985. Use of particulate limestone or oyster shell in the dietary regimen of White Leghorn hens. Poult. Sci., 64: 1750-1762.
24. Roland, D.A.Sr., M.M. Bryant, R.H.W. Abon and J. Self, 1996. Influence of calcium and environmental temperature on performance of first-cycle (Phase 1) commercial Leghorns. Poult. Sci., 75: 62-68.
25. Miller, P., 1993. Reducing (controlling) egg weight. California Poultry Letter, pp: 4-8.
26. Pelicia, K., E.A. Garcia, A.B.G. Faitarone, A.P. Silva, D.A. Berto, A.B. Molino and F. Vercese, 2009. Calcium and available phosphorus levels for laying hens in second production cycle. Revista Brasileira Ciencia Avicola, 11: 39-49.
27. Lichovnikova, M., 2007. The effect of dietary calcium source, concentration and particle size on calcium retention, eggshell quality and overall calcium requirement in laying hens. Br. Poult. Sci., 48: 71-75.
28. Rao, K.S. and D.A. Roland Sr., 1989. Influence of dietary calcium level and particle size of calcium source on *in vivo* calcium solubilization by commercial Leghorns. Poult. Sci., 68: 1499-1505.
29. Zhang, B.F. and C.N. Coon, 1997. The relationship of calcium intake, source, size, solubility *in vitro* and *in vivo* and gizzard limestone retention in laying hens. Poult. Sci., 76: 1702-1706.